



Nutrient limitations induced by drought affect forage N and P differently in two permanent grasslands



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ABSTRACT

Drought events can strongly affect ecosystem functioning by modifying relationship between plants, microbes and soil chemistry, with consequent impacts on nutrient cycling. However, the potential impacts of a soil moisture reduction on the nitrogen (N) and phosphorus (P) cycling in grasslands remain poorly understood, especially in regard to forage production.

To fill this knowledge gap, a drought experiment was carried out using rainout shelters in two permanent grasslands, characterized by similar vegetation communities but contrasted soil nutrient limitations. Drought treatments were applied during two months, either when plant growth was highest (Early-season drought) or after the peak of biomass production (Late-season drought). Dry matter production, forage N status (NNI) and P content as well as N and P contents in microbial biomass and soil were determined.

Both early and late-season drought significantly reduced soil moisture during the vegetation growth period. Forage yield was also reduced by drought, but only when it occurred late in the season. Using a structural equation model, we showed that soil moisture reduction had a direct effect on forage N status, suggesting that water shortage induced lower transpiration and water fluxes. Soil moisture reduction also affected forage P by reducing the availability of soil P. However, other mechanisms played a larger role and were site-specific. At the more fertile site, reduction in soil moisture directly impaired forage P, suggesting that water stress mainly resulted in lower diffusion rates to roots, while at the less fertile site, an indirect reduction of forage P through a pathway implying microbes (decrease in microbial P) was detected.

Our results suggest that the two grasslands suffered mainly from water shortage *per se*, but also from drought-induced nutrient deficiency (mainly P), which amplified yield losses and further decreased forage quality. Overall, our findings emphasize the need for further research on the plant-soil-microbe system functioning, in order to secure a sustainable and resilient forage production in the context of climate change.

1. Introduction

Drought events are recurring phenomena, which are predicted to increase in frequency and intensity over the next few decades due to climate change (IPCC, 2013). Water shortage is recognised as one of the biggest threat to agriculture (Lesk et al., 2016), with important economic consequences. Grasslands, as core areas for forage production

worldwide, are particularly affected by extended periods of drought. Therefore, understanding how grasslands respond to extended drought periods is a major concern, especially in areas dedicated to high added value food products (e.g., cheese with protected designation of origin). In particular, drought timing is an important factor affecting grassland communities (Chou et al., 2008; Craine et al., 2012; De Boeck et al., 2011), but it remains poorly studied. Moreover, since drought events

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impact both above and belowground components of grassland ecosystems (Bardgett et al., 2013), changes in forage quantity and quality must be investigated with particular consideration of the effects of soil moisture reduction on the complex interactions among soil, microbes and plants.

In the soil, water scarcity can alter diverse processes of the nitrogen (N) cycle, but the effects are strongly site-specific (i.e., dependent on agricultural management, soil and vegetation type), thus reflecting the complexity of the processes. For example, a reduction in gross N mineralisation as a result of drought has been reported in a heathland (Larsen et al., 2011), whereas no effects were observed in a *Trisetum flavescens*-dominated meadow (Fuchslueger et al., 2014). Nitrification rates responded to temperature (Grundmann et al., 1995) and, to a lesser extent, to precipitation treatments (Auyeung et al., 2013; Hartmann et al., 2013). Furthermore, depending on the severity of the drought stress, water shortage can impair symbiotic nitrogen fixation (Hofer et al., 2017). With respect to phosphorus (P), its bioavailability is also influenced by many factors, such as P sources, pH and soil moisture (Dijkstra et al., 2015; Shen et al., 2011). The P cycling can be potentially altered under drought through lower desorption (Belnap, 2011) and lower diffusion rates (Lambers et al., 2008). Since plant and soil microbes have different sensitivities to restriction in N and P availability (Dijkstra et al., 2015), drought-induced change in N and P cycles can perturb plant-microbes interactions. While certain plant groups show stoichiometric N:P flexibility through symbiotic interactions with mycorrhizal fungi (Mariotte et al., 2017), microorganisms rather adapt through changes in the structure of the community (Čapek et al., 2016; Darcy et al., 2018; Tischer et al., 2014).

Forage production is affected by drought either directly, through plant physiological processes such as stomata closure, reduction of the photosynthesis activity, modified allocation of resources in plant parts (Chaves et al., 2002), or indirectly, through reduced soil nutrient availability (He and Dijkstra, 2014). Disentangling direct effects of drought on plants (due to water shortage *per se*) from the indirect effects linked to soil nutrient availability and plant nutrition is a difficult task. Since N and P are often limiting nutrients for plant growth, the processes involving soil N and P are of key importance. As long as soil nutrient supply exceeds plants demand, there is no drought-induced nutrient limitation. In contrast, indirect effects on growth arise when nutrient demand by plants is higher than their availability in soil (Gonzalez-Dugo et al., 2010). This highlights the importance of soil characteristics, particularly the N and P availability and the N:P ratio as potential drivers of grasslands response to drought perturbations.

A “critical N concentration” can be used to describe the relationship between plant N and biomass accumulation (Lemaire et al., 1984). It represents the minimal level of N content (at a given plant biomass) for achieving the maximum growth rate. The nitrogen nutrition index (NNI) is defined as the ratio between the actual (measured) and the critical N concentration in plant (Lemaire and Gastal, 1997; Lemaire et al., 1989). The NNI provides information on the nutrition status of the forage and may be used to identify drought-induced grassland N limitation. The validity of NNI has been assessed in a wide range of conditions and it can be used irrespectively of botanical composition (Duru et al., 1997). Additionally, nutrient limitations for plant growth can also be estimated from vegetation N:P ratio (aboveground material), where N:P ratio below 10 reflects N limitation, while N:P ratio above 20 suggests P limitation (Güsewell, 2004).

Only few studies addressed the effect of drought on plant-soil relationship under field conditions (Buttler et al., 2019; Dijkstra et al., 2015; Mariotte et al., 2017). Indeed, to our knowledge, this is the first study on permanent grasslands describing simultaneously the impact of drought with both an ecological and an agronomic perspective and considering the overall plant-soil interactions. Here we used an experimental set-up to simulate different precipitation regimes in two permanent grasslands characterized by comparable vegetation but different soil nutrient limitations (a more fertile vs a less fertile soil). We

applied the same drought treatments at both sites, either during (i.e., early-season drought) or after (i.e., late-season drought) the peak of plant growth, and surveyed the responses of N and P cycling to drought in the plant-soil-microbe system just after the periods of water stress and during the subsequent recovery period. We expected (i) forage quantity to decrease during the drought periods, with more pronounced effects early in the season, when the peak of growth occurs and water demand is higher. Moreover, we hypothesized that (ii) forage yield and quality would be reduced through direct but also indirect effects of water limitation (i.e., decrease of soil N and P availability). Our last hypothesis was that (iii) forage yield and quality would be reduced more at the less fertile site due to increased nutrient limitation and competition for resources between plants and microbes under drought stress.

2. Materials and methods

2.1. Experimental sites

The experiment was conducted from spring 2015 to fall 2016 at two permanent grasslands sites and results shown in this manuscript correspond to the data collected during the growing season in 2016. Both sites were located in the Swiss Jura Mountains: Site 1 at Chésérèx (N 46°24', E 6°10') and Site 2 at Saint-George (N 46°30', E 6°15'), respectively at 540 and 940 m a.s.l. Climate at the two sites is sub-oceanic with average annual precipitation of 1050 and 1290 mm and average annual temperatures of 10.4 °C and 7.6 °C (averaged 1981–2010, MeteoSwiss, Zurich, Switzerland). Average precipitation during the period of our experiment (April to September) is 441 mm at Site 1 and 682 mm at Site 2, and average daily temperatures are 16.0 °C at Site 1 and 13.5 °C at Site 2 (averaged 1981–2010 period, MeteoSwiss, Zurich, Switzerland). Nonetheless, the Jura Mountains may undergo hot and dry periods. The botanical composition of the two sites was similar and dominated by *Lolium perenne* L., *Dactylis glomerata* L., *Poa pratensis* L., *Trifolium repens* L. and *Taraxacum officinale* Weber ex F.H.Wigg. aggr., which together accounted for at least 80% of total plant biomass. Both sites were fertilised according to the Swiss recommendations (Sinaj et al., 2009) with an organic manure (5.2% organic nitrogen and 4.4% phosphate), in split applications, half in October and half in March. The total fertiliser application rate during the experiment was 150 kg N ha⁻¹ and 125 kg P ha⁻¹.

Soils at Site 1 and 2 are both classified as cambisols (IUSS Working Group WRB, 2006) but are quite different regarding depth, organic matter (OM) content, N and P availability. Site 1 has a deeper soil (90 cm) and is characterized by 19.8% clay, 41.2% silt and 38.9% sand, a pH of 5.8 and 4.7% OM. Average soil available N (dissolved inorganic nitrogen) is 16 mg kg⁻¹ and available P (Olsen method) is 9 mg kg⁻¹. Forage (aboveground material) has an average N:P ratio of 8.9 over the growing season, therefore site 1 tends to be N-limited for plants (i.e., plant N:P < 10, Güsewell, 2004) despite very low soil P availability. Overall, soil at site 1 was considered less fertile than soil at site 2. Site 2 has a shallower soil (45 cm depth) and is characterized by 36.3% clay, 41.5% silt and 24.2% sand, a pH of 7.5 and 8.5% OM. Average soil available N is 27 mg kg⁻¹ and available P is 51 mg kg⁻¹. Forage (aboveground material) has an average N:P ratio of 6.8 over the growing season. Therefore, site 2 tends also to be N-limited for plants, even more than site 1, but has very high soil P availability. Overall, soil at site 2 was considered as fertile.

2.2. Experimental design and drought treatments

An identical drought experiment with three drought treatments was carried out at the two grassland sites: Control (no drought), early-season drought event (hereafter called ‘Early’) and late-season drought event (hereafter called ‘Late’). At the beginning of the experiment, five replicated rainout shelters (length: 12 m; width: 6.4 m, height: 3 m,

Filclair, Numeris 6.40, Vennelles, France) covered with a transparent plastic film (180 μm , transparent M42, Filclair, Vennelles, France) were established at each site. Along the longitudinal sides, the lower part remained uncovered up to 1 m, whereas along the short side the shelters were open, allowing free air circulation, but a thin (mosquito) netting was placed on the upper part to prevent rain from blowing in. Rainwater runoff was canalised away from the covered surface thanks to plastic pipes. Three plots of 4 m $\times$ 0.9 m (separated by 80 cm) corresponding to the three drought treatments (*i.e.*, Control, Early and Late) were randomly installed under each rainout shelter with a buffer distance of at least 80 cm from the borders of the shelters.

Control plots were watered according to the average precipitation of the last thirty years at each site (*i.e.*, 441 mm at Site 1 and 682 mm at Site 2 over the 6 months of the experiment). Drought plots simulated rainfall conditions according to the intermediate scenario of climatic models in our study region (CH2011, 2011). The Early drought treatment consisted in a reduction of precipitation that occurred during two months centred on the peak of plant growth (from about mid-April to mid-June). For the Late drought treatment, the reduction in precipitation occurred for two months after the Early drought (about mid-June to mid-August). During the respective drought event, plots only received 30% of the water added in the control plots. Outside the Early or Late drought-induced periods, plots received the same amount of water as the control plots until the end of the growing season, which corresponded to the two months after the end of the late-season drought (recovery period; about August to September). Watering was done manually by means of a flow controller and a custom-made watering stick, which was moved along the plots to simulate a regular rain. Watering occurred every 2–3 days in control plots to simulate the rainfall frequency of the region (*i.e.*, about 11 rainy days per month during the growing season), and every 4–5 days in drought plots to simulate 50% decrease in rainfall frequency, which is expected to occur simultaneously with precipitation reduction (CH2011, 2011). The sites received ambient precipitation for the rest of the year (rain-out shelters were uncovered).

During the experiment, all plots were managed according to the forage conservation regime (hay making) typical for the region, *i.e.*, plots were mowed three times during the season. The forage harvests are referred to as T1, T2 and T3; T1 corresponding to the first cut (end of Early drought), T2 to the second cut (end of Late drought) and T3 to the third cut (end of the recovery period). More detailed information about the experimental design is available in Vitra et al. (2019).

2.3. Soil moisture and PAR measurements

Soil volumetric moisture content was measured once a week with a time domain reflectometer (FieldScout TDR 100 Soil Moisture Meter, Spectrum Technologies, USA) for the top 15 cm of the soil. Three randomly located moisture measurements per plot were averaged. Photosynthetically active radiation (PAR) was also measured (PYR Solar Radiation Sensor, Decagon, München, Germany) under the shelters and outside. The transparent foil induced a decrease of about 20% of the PAR (compared to the measurement outside), which was never limiting for plant growth based on biomass comparison with non-covered plots.

2.4. Botanical survey

The botanical composition was determined three times before mowing, at T1, T2 and T3 described above, following the pin-point method of Daget and Poissonet (1971). Surveys were completed in all the 30 plots (15 per site) by setting up within each plot four parallel transects of 4 m, in order to survey systematically the whole plot surface (grid of squares, 20 cm $\times$ 20 cm; 80 points per plot). At each point, a present species was recorded only once. The relative cover of each species was calculated by dividing the number of presences of the

species by the total number of records of all species along the four transects within a plot (Iussig et al., 2015). We then determined the relative cover of the following functional groups: grasses, legumes and forbs.

2.5. Forage sampling and chemical analysis

Forage was cut at T1, T2 and T3 using a motor mower (*c.* 5 cm above the soil level) and the fresh aboveground biomass harvested in each plot was weighed and sampled with a hay probe. The samples were divided in two subsamples: the first one for dry matter (DM) determination and the second one for chemical analyses. All subsamples were oven-dried at 60 °C for 72 h after harvest; subsamples for DM determination were then dried three additional hours at 105 °C. The subsamples for chemical analysis were ground and sieved through a 1 mm mesh screen (Brabender, Germany), and analysed for N using near-infrared spectroscopy (Büchi NIRFlex N-500; BÜCHI Labortechnik AG, Switzerland). Forage nitrogen nutrition index (NNI) was calculated according to following equations:

$$\text{Forage NNI} = \text{N measured} / \text{N critical (Lemaire and Gastal, 1997);}$$

$$\text{N critical} = 4.8(\text{DM})^{-0.32} \text{ (Lemaire et al., 1984);}$$

where 'N measured' corresponds to the forage aboveground N concentration (%) and 'N critical' to the minimal N concentration (at a given biomass) for achieving maximum growth rate. DM represents the biomass (for DM > 1 Mg). NNI values were adjusted according to the percentage of legumes, in order to account for biological nitrogen fixation (Cruz et al., 2006). Forage P was analysed from dry ash using an inductively coupled plasma optical emission spectrophotometer (IPC-OES, Optima 7300 DV, Perkin-Elmer, Switzerland).

2.6. Soil sampling and chemical analysis

In every plot, soil was sampled at T1, T2 and T3 by collecting 15 spatially random distributed soil cores (diameter 1.2 cm, depth 15 cm), which were pooled together and homogenized. Soil samples were analysed for available N and P and microbial biomass N and P. Dissolved inorganic nitrogen (DIN, hereafter called soil N) was determined by measuring and summing ammonium (NH_4^+) and nitrate (NO_3^-) concentrations, after extraction of 5 g fresh soil with 30 ml 1 M KCL, using an automated analyser (AA3 HR Autoanalyser, Seal Analytical, UK).

For the determination of microbial biomass N, pairs of *c.* 5 g of fresh soil were weighed and one sample from each pair was immediately extracted with 25 ml 0.5 M K_2SO_4 , whereas the other sample was first fumigated with chloroform for 1 day to kill microbes and then extracted with the same solution as for the unfumigated sample. Total N concentrations in fumigated and unfumigated samples were analysed by a TOC/TN analyser (Total organic carbon analyser TOC-V, Shimadzu, Japan). To determine the soil available P and the microbial biomass P, pairs of *c.* 3 g of fresh soil (fumigated and unfumigated) were extracted with 40 ml of 0.5 M NaHCO_3 . Phosphorus concentrations (hereafter called 'soil P') were analysed colorimetrically (spectrophotometer at 890 nm) using the ammonium molybdate reagent (Olsen et al., 1954). Microbial biomass N and P were estimated as the differences between the concentration of N and P in fumigated and unfumigated sample using an extractability factor of 0.54 for N (Brookes et al., 1985) and 0.4 for P (Brookes et al., 1982). Microbial and soil N and P were expressed as mg kg^{-1} dry soil (oven-dried at 105 °C for 24 h).

2.7. Statistical analyses

All analyses were carried out with R version 3.4.4 (R Development Core Team, 2017). Due to major soil differences between Site 1 and Site 2, we ran analyses for each site separately. Dataset was log-transformed (natural logarithm) prior to statistical analysis to improve and/or comply with the assumptions of normality and homoscedasticity.

Drought treatment effects (Control, Early and Late) over the growing season (time of harvest T1, T2 and T3) on soil moisture, forage yield, forage NNI, forage P, soil N, soil P, microbial N and microbial P were analysed with a linear mixed-effects model (lme) taking into account the data structure with repeated measurements at the plot level (i.e., non-independence of the subject's residuals). Thus, the effects of 'drought', 'time' (as repeated measurements) and their interaction were set as fixed factors with 'plot' nested into 'block' as random factor.

Overall, drought treatment (Control, Early, Late) had strong significant effects on soil moisture (see results) but only few effects were detectable on the other variables (soil N, microbial N, forage NNI, soil P, microbial P, forage P; Fig. A.1, A.2 in Appendices), with the exception of forage P at Site 2. Therefore, despite originally creating a factorial drought treatment based on precipitation reduction, we further analysed and interpreted the range of the effects produced by water stress on the forage, microbial, and soil variables by using soil moisture (e.g., continuous variable) as explanatory variable in subsequent statistical analyses. With this goal, the effects of soil moisture (i.e., TDR measurement) over the vegetation growing season (T1, T2 and T3) on forage yield, forage NNI, forage P, soil N, soil P, microbial N and microbial P were analysed with a linear mixed-effects model (lme) with 'soil moisture' and 'time' as fixed factors and 'plot' nested into 'block' as random factor, as well as with linear regressions.

Complex interactions among the effects of drought on soil moisture, soil nutrients availability, forage and microbial nutrients' concentrations were analysed through structural equation modelling (Grace et al., 2014). We used a path analysis approach, a particular case of structural equation modelling involving only quantitative variables, to test for the effect of soil moisture reduction resulting from drought manipulation on N and P cycling in the plant-microbe-soil system. Using *a priori* knowledge, we built a network of causal relationships among measured variables for N- and P-cycling (Fig. A.3). Models were run separately for Site 1 and Site 2 and fit using maximum likelihood estimation with robust SE and Satorra-Bentler scaled test statistic in R with the LAVAAN package (Rosseel, 2012). Then, the successive full models were simplified by step-wise exclusion of variables with non-significant weights and non-significant covariance, as estimated by Akaike information criterion, until minimal adequate models showing specific linkages remained. Z-statistic was used to determine the significance of each pathway. Final model fits were assessed with chi-square test ($P > 0.05$), standardized root mean square residual index (SRMR < 0.1) and comparative fit index (CFI > 0.95) (Grace et al., 2010).

3. Results

3.1. Drought treatment effects on soil moisture

At both sites, the drought treatments significantly affected soil moisture (Fig. 1a,b). As expected, a reduction of soil moisture was detected during both drought periods, i.e., in the Early drought plots at T1 and in the Late drought plots at T2. While soil moisture recovered in Early drought plots after the drought period (T2 and T3), a legacy effect of Late drought was observed during the recovery period (T3, significant at Site 2 and trend at Site 1). Overall, the relative effect of Late drought treatment was slightly higher at Site 2 than at Site 1 (i.e. soil moisture decrease relative to control plots, Fig. 1a,b).

3.2. Drought treatment effects on plant community composition and forage yield

The botanical composition showed a clear seasonal pattern at both sites with a decrease in relative grass cover throughout the season and an increase in relative forb cover (Fig. A.4). Drought had only limited effects on functional groups (i.e., undifferentiated effect between groups), and its impacts were independent of the period (no significant 'drought x time' interaction). Early-season drought induced a decrease

in relative grass cover at Site 2, while Late-season drought induced a decrease in relative forb cover at both sites. The relative legume cover was only affected by drought at Site 2, with an increase in plots that experienced Early-season drought. Apart from these slight changes in the proportion of functional groups under drought, the botanical composition remained relatively constant at both sites (see also Vitra et al., 2019).

Forage yield significantly differed among the three harvest times and showed a decrease along the season (Fig. 2a,b). At both sites, forage yield was only affected by drought at T2. Indeed, forage yield decreased during Late drought, with a stronger decrease at Site 2 (Fig. 2b, T2, L, -68%,) compared to Site 1 (Fig. 2a, T2, L, -32%,), but recovered at the end of the growing season (T3). Additionally, forage yield also decreased at the more fertile site in plots that previously experienced Early-season drought (Fig. 2b; T2, E, -22%). These effects were confirmed when 'soil moisture' was used as a continuous explanatory variable. Indeed, we observed a highly significant 'soil moisture x time' interaction (Table 1) at both sites, which is due to the decrease in forage yield with decreasing soil moisture at T2 (significant linear regression for T2; Site 1: $P < 0.01$, $R^2 = 0.59$; Site 2: $P < 0.001$, $R^2 = 0.84$). Moreover, independently of soil moisture, forage yield was overall positively correlated with soil P (data not shown, Site 1: $P < 0.001$, $R^2 = 0.35$; Site 2: $P < 0.001$, $R^2 = 0.38$) but less influenced by soil N (data not shown, Site 1: $P = 0.18$; Site 2: $P = 0.04$, $R^2 = 0.14$).

3.3. Effects of drought on soil, microbial and forage nitrogen

At both sites, soil N and forage NNI were significantly affected by 'soil moisture' and 'time', while microbial N was affected by 'soil moisture' and 'time' at Site 2 and only by 'time' at Site 1 (Table 1). No significant 'soil moisture x time' interactions were found for all N-related parameters, thus highlighting consistent effects of soil moisture reduction independently of the time of measurement. Soil N slightly increased at Site 1 (Fig. 3a) while it strongly decreased at Site 2 (Fig. 3d) with decreasing soil moisture. At both sites, forage NNI significantly decreased with soil moisture reduction (Fig. 3c,f), whereas microbial N was not impacted (Fig. 3b,e).

3.4. Effects of drought on soil, microbial and forage phosphorus

At both sites, soil P, microbial P and forage P were significantly affected by 'soil moisture' and 'time' (Table 1). Significant 'soil moisture x time' interaction was only found for forage P at Site 2, which is due to different amplitude of soil moisture effect rather than shifts in effects among the three periods of harvest (i.e., forage P always significantly decreased with soil moisture reduction at T1, T2, and T3, see also Fig. A.2f). At both sites, soil P (Fig. 4a,d), microbial P (Fig. 4b,e) and forage P (Fig. 4c,f) significantly decreased with soil moisture reduction.

3.5. Nitrogen and phosphorus in the plant-soil-microbe system

The fitting parameters of the minimal adequate path analysis models, linking the plant, soil and microbial pools of either N or P, indicate good fit for all models (Fig. 5a,b). At both sites, the decrease in soil moisture had direct strong negative effect on forage nitrogen nutrition index (Fig. 5a, path = -1.01^{***} at Site 1, and path = -0.92^* at Site 2). No significant indirect effects of soil moisture reduction on forage NNI were found at Site 1. By contrast, the decrease in soil moisture at Site 2 negatively impacted forage NNI through a reduction in soil N (indirect effect path, $-2.25^{**} \times 0.25^{**} = -0.56$). Microbial N was not directly affected by soil moisture reduction at both sites. At Site 1, a decrease in soil moisture marginally increased soil N (path = 0.91), which subsequently increased N pool in microbes (path = 0.50^{***}) rather than in plants. Drought differentially affected the P parameters in both sites (Fig. 5b). Thus, a reduction in soil moisture had a strong direct negative effect on forage P at Site 2 (path = -1.13^{**}) but not at

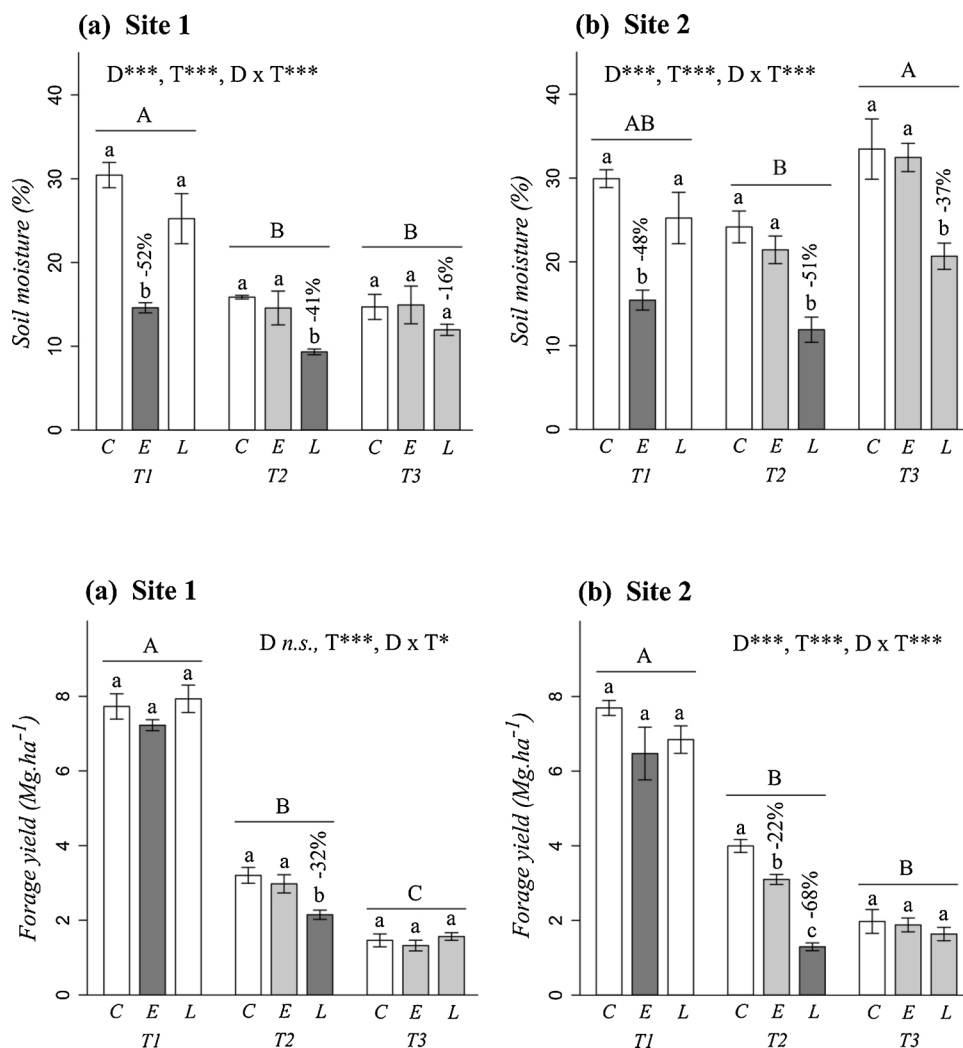


Fig. 1. Soil moisture (± SE) at (a) Site 1 and (b) Site 2 in the different drought treatments (C: Control, E: Early-season drought, L: Late-season drought) during the growing season (Time: T1, T2 and T3). Dark grey bars highlight when plots were experiencing drought, either Early-season (T1, E) or Late-season drought (T2, L) treatment, while grey bars highlight plots that were previously subjected to drought. Mean decreases in soil moisture in drought plots compared to their respective control plots are indicated in percent above the bar when relevant. Significance level of the effects of drought (D), time (T) and their interactions (D x T) are indicated by *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Significant differences between times of measure are indicated by different capital letters, while significant differences among drought treatments within each time of measure are indicated by different lowercase letters.

Fig. 2. Forage yield (± SE) at (a) Site 1 and (b) Site 2 in the different drought treatments (C: Control, E: Early-season drought, L: Late-season drought) during the growing season (Time: T1, T2 and T3). Dark grey bars highlight when plots were experiencing drought, either early-season (T1, E) or Late-season drought (T2, L) treatment, while grey bars highlight plots that were previously subjected to drought. Mean decreases in forage yield in drought plots compared to their respective control plots are indicated in percent above the bar when relevant. Significance level of the effects of drought (D), time (T) and their interactions (D x T) are indicated by *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Significant differences between times of measure are indicated by different capital letters, while significant differences among drought treatments within each time of measure are indicated by different lowercase letters.

Table 1

Effects of soil moisture (SM), time (T) and their interaction (SM x T) on forage yield, soil N, microbial N, forage nitrogen nutrition index (NNI), soil P, microbial P and forage P at Site 1 and Site 2. F and P values correspond to the statistical output of linear mixed-effects models (lme).

	Site 1						Site 2					
	SM		T		SM x T		SM		T		SM x T	
	F	P	F	P	F	P	F	P	F	P	F	P
Forage yield	286.93	< 0.001	217.62	< 0.001	4.54	0.03	10.87	< 0.01	155.30	< 0.001	10.61	0.001
Soil N	5.88	0.03	14.28	< 0.001	0.47	0.64	40.17	< 0.001	29.61	< 0.001	2.75	0.10
Microbial N	0.04	0.84	3.80	0.04	0.45	0.65	4.93	0.04	6.94	< 0.01	1.24	0.31
Forage NNI	26.12	< 0.001	6.09	< 0.01	0.77	0.48	27.52	< 0.001	8.65	< 0.01	3.77	0.05
Soil P	5.85	0.03	4.20	0.03	1.04	0.37	12.12	< 0.01	27.51	< 0.001	0.72	0.50
Microbial P	21.22	< 0.001	10.42	< 0.001	0.52	0.60	26.16	< 0.001	22.15	< 0.001	1.79	0.20
Forage P	26.76	< 0.001	9.97	0.001	1.66	0.22	76.33	< 0.001	11.05	< 0.001	4.47	0.03

Site 1. At Site 2, forage P was also indirectly impacted by soil moisture reduction through changes in soil P, while such effects were limited at Site 1. At Site 1, the main effect of soil moisture reduction on forage P was indirect and mediated by microbes. Indeed, soil moisture reduction strongly reduced microbial P (path = -2.86**) with subsequent significant effects on forage P (indirect effect path, -2.86** × 0.15* = -0.43).

4. Discussion

4.1. Forage yield reduction only occurred during late-season drought

Contrary to our hypothesis, plant growth remained sustained during the first drought period, even with only 30% of the 'normal' precipitation. Indeed, despite a high evaporative demand during the early-season drought, soil water reserve proved to be sufficient to ensure plant growth, likely explaining the lack of the expected effect on

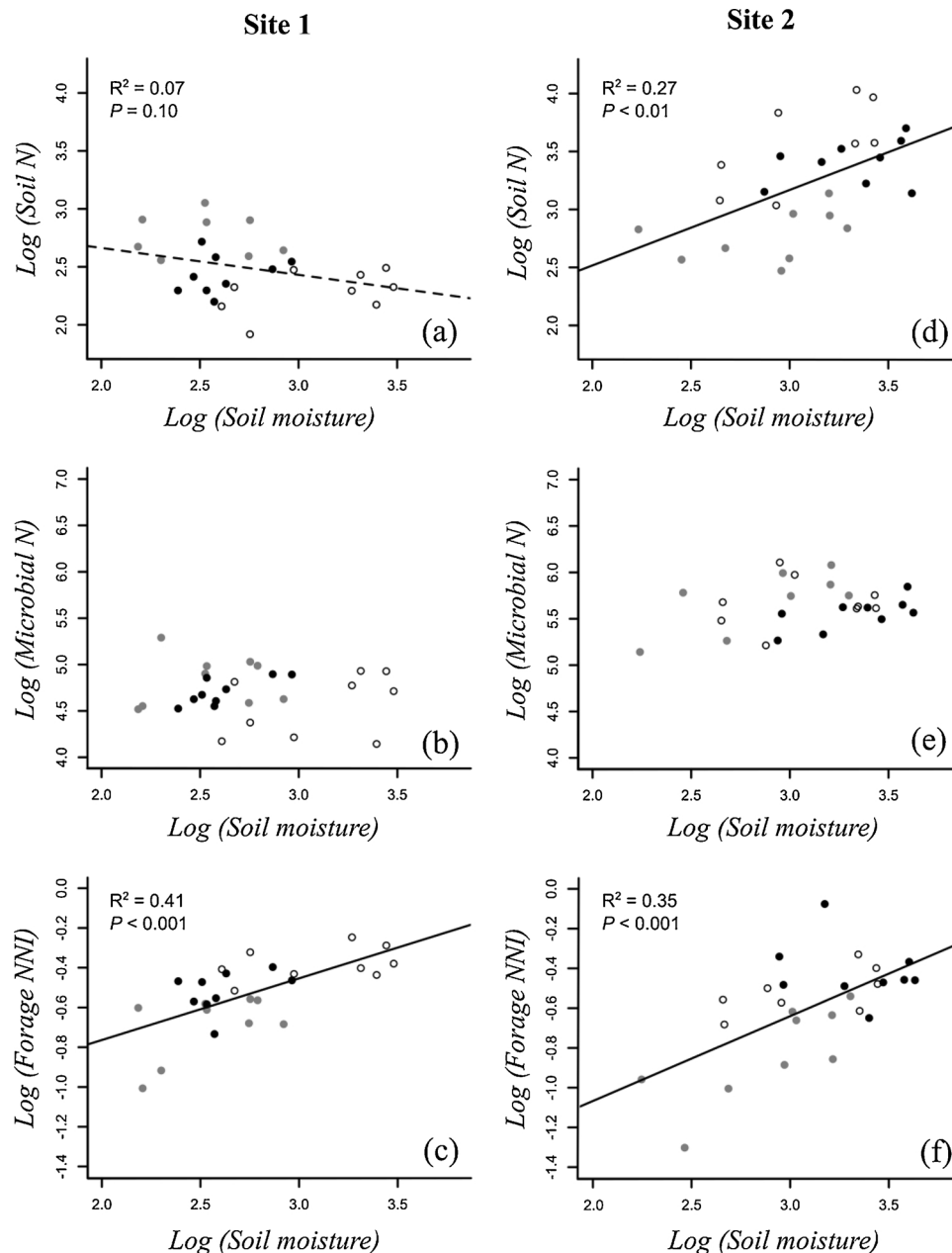


Fig. 3. Linear relationships between N parameters in soil and plant and soil moisture at Site 1 and Site 2; (a, d) soil N (in mg. kg^{-1}), (b, e) microbial N (in mg. kg^{-1}), and (c, f) forage NNI. Empty circles highlight data collected at T1, while grey and black dots correspond to data collected at T2 and T3, respectively. Data are log-transformed and black lines highlight significant regressions while dashed line indicate marginally significant regression.

biomass production. Later in the season, the water reserve steadily decreased, reaching the lowest values at T2. As previously reported (Craine et al., 2012; De Boeck et al., 2011), the timing of stress is critical regarding the response to water shortage, with stronger effects in summer compared to spring. Since we surveyed soil moisture in the upper 15 cm, the values might not fully reflect total available water for plants. Although most of the fine roots of the permanent grasslands lie in the upper 15 cm (Prechse, 2013), it must be taken into consideration that some species may develop their root system in deeper layers, such as *D. glomerata* (Farjon, 2011), and this can happen for a given species as a result of drought (Buttler et al., 2019). For this reason, during the first period of drought, plants might have been still able to access water reserves at deeper soil layers, in contrast to the second period that showed to be more water-depleted. Recent work has underpinned the importance of atmospheric demand (vapour pressure deficit, VPD) for ecosystems functioning, such as carbon and water fluxes (Novick et al.,

2016). An increase in vapour pressure deficit, combined with soil moisture reduction, has a much more pronounced effect on primary production than soil water restriction alone (Eamus et al., 2013). In this experiment, vapour pressure deficit was lower during the early-season drought, compared to the late-season drought (Site 1: 0.5 and 0.8; Site 2: 0.3 and 0.6, respectively). These differences in VPD may have enhanced the effect of soil moisture reduction, and could partially explain the fact that yield losses were only observed during the second drought period.

4.2. Forage yield reduction is primarily due to water deficit, but also to the reduction in soil P availability

As the botanical composition remained quite stable during the experiment, we can assume that almost all changes in forage production were caused by water shortage, either through direct effect (i.e., water

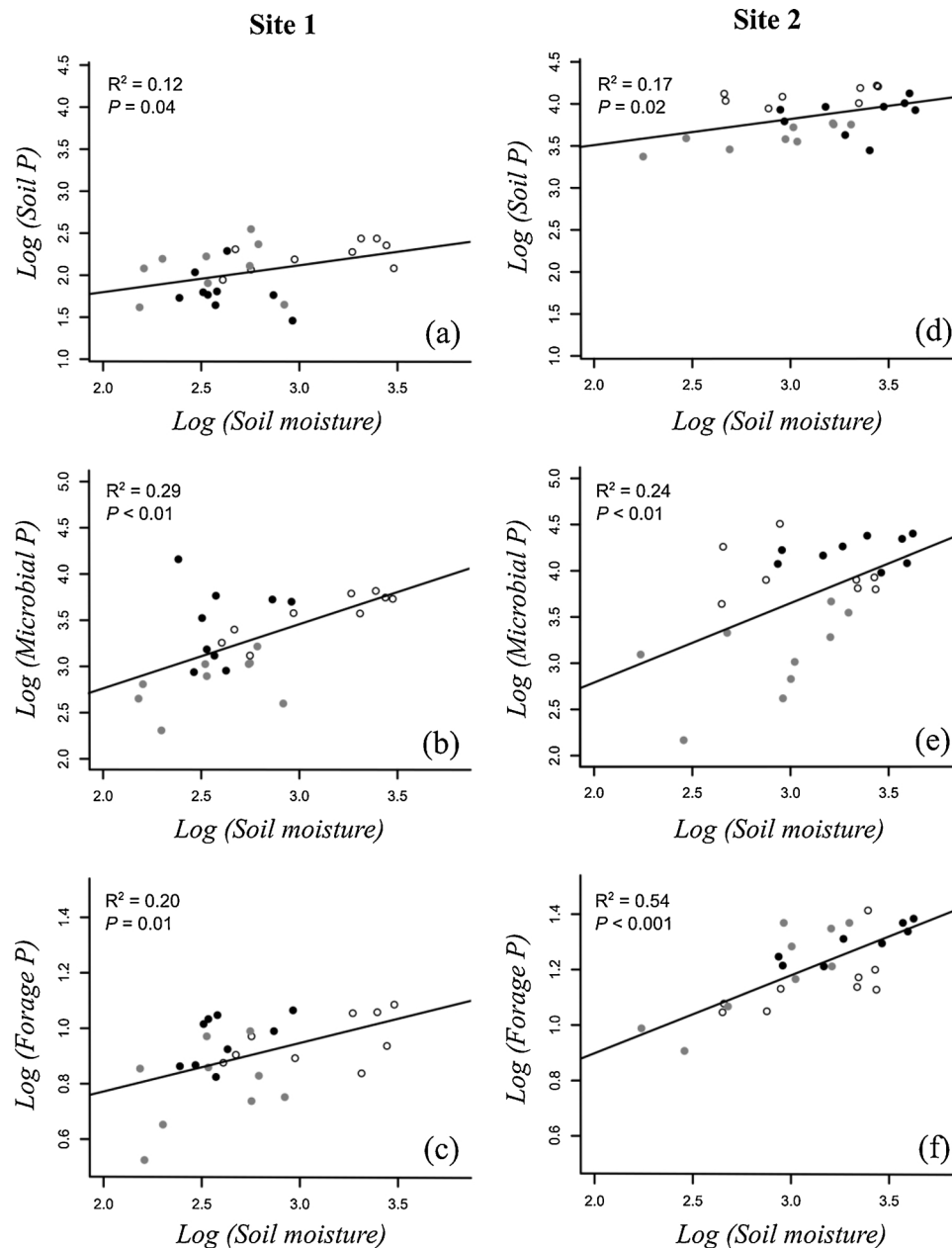


Fig. 4. Linear relationships between P parameters in soil and plant and soil moisture at Site 1 and Site 2; (a, d) soil P (in $\text{mg} \cdot \text{kg}^{-1}$), (b, e) microbial P (in $\text{mg} \cdot \text{kg}^{-1}$), and (c, f) forage P (in $\text{mg} \cdot \text{g}^{-1}$). Empty circles highlight data collected at T1, while grey and black dots correspond to data collected at T2 and T3, respectively. Data are log-transformed and black lines highlight significant regressions.

deficit for plants) or through indirect drought-induced nutrient deficiency (*i.e.*, restricted availability and diffusivity/movement of nutrient in soil water). However, it is difficult to make a clear distinction between these two driving forces, since there is generally an interplay between these processes (Gonzalez-Dugo et al., 2010). The interaction between P supply and water availability on plant development has been poorly studied, and generally in pot experiments (see review of Suriyagoda et al., 2014). Jupp and Newman (1987) observed that a mild water stress almost stopped P uptake of *Lolium perenne*, which was likely due to a reduction in P diffusion in soil, rather than to an inability of the roots to take up P. Under low moisture (*i.e.*, water holding capacity of 30% compared to 75%), P uptake of wheat plants was strongly reduced (46–66%, depending on the level of P supply), while shoot biomass diminished between 38 and 60% (He et al., 2002). The results of our study are in line with these observations and confirm our second hypothesis. They point primarily to direct water limitation, reinforced

by lower P availability, since yield was positively correlated with soil moisture and soil P availability at both sites. By contrast, soil N limitation seems to have played a secondary role.

4.3. Higher forage yield losses observed at the more fertile site

Forage yield losses recorded during the late-season drought were higher at the more fertile site, with a higher reduction in soil moisture, which is opposed to our third hypothesis, at least for what impacts yield. The water reserve in soil depends primarily on its texture and depth (Allen et al., 1998). According to our water balance calculations (Allen et al., 1998), the available water was about 130 mm at Site 1 (80 cm depth; 20% clay and 41% silt) and only 60 mm at Site 2 (45 cm depth; 36% clay and 41% silt). Furthermore, the soil at Site 2 had a more skeletal structure. These differences may explain the stronger response to drought observed at Site 2. Considered on an annual basis,

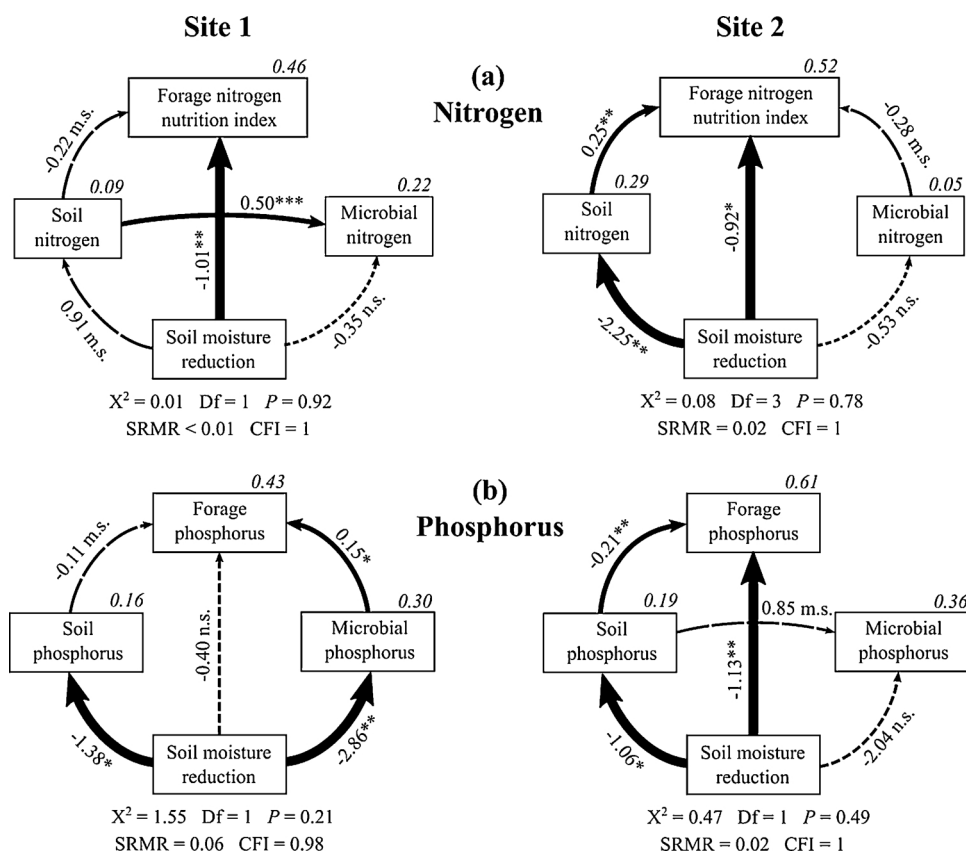


Fig. 5. Minimal adequate structural equation model for the effects of soil moisture reduction on the linkages between (a) N in soil and plant parameters and (b) P in soil and plant parameters at Site 1 and Site 2. The model shows the direct effect of soil moisture reduction on forage NNI (or forage P) as well as its indirect effect through changes in soil and microbial N (or P). Arrows show relationships (pathways) between variables and numbers next to arrows show standardized parameter estimates (i.e., standardized regression weights). Square multiple correlations (R^2 values) for the predicted/dependent factors are given on the boxes. CFI: comparative fit index; SRMR: standardized root mean residual. Significance level of the paths are indicated by *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, m.s. $P < 0.10$, n.s. $P > 0.10$.

water shortage had no effect on the yield at Site 1 while a reduction of 16% and 28% was observed at Site 2 for early- and late-season drought, respectively (data not shown). These annual losses are in the same order of magnitude than those observed in permanent grasslands, with stress events qualified as “extreme” (Gilgen and Buchmann, 2009; Kahmen et al., 2005; Mariotte et al., 2013; Vogel et al., 2012). Surprisingly, we did not observe an overcompensation of plant growth after drought, as reported in previous papers (Hofer et al., 2017).

4.4. Drought effects on forage nutrient content depend on soil nutrient availability

Drought events can impact grassland N and P contents by modifying soil N and P availability (Belnap, 2011; Lambers et al., 2008) and N and P uptake by plants and microbes (Dijkstra et al., 2015; Jiao et al., 2016). As a result, drought-induced changes in both N and P cycling can have strong effects on forage nutrient content. The effects of drought on forage nitrogen nutrition index (NNI) have been largely discussed (e.g., Gastal et al., 2015; Gonzalez-Dugo et al., 2010; Onillon et al., 1995) and are associated either to reduced N mineralisation, the need for plants to extract water from deeper soil layers with less available N (Lemaire and Denoix, 1987) and/or a reduced N demand at a given plant biomass (Errecart et al., 2014; Gonzalez-Dugo et al., 2012). In our experiment, soil N availability was significantly affected by soil moisture reduction but responded differently between the two sites. Indeed, we observed a strong decrease in soil N availability with decreasing soil moisture at Site 2, while it slightly increased at Site 1. Such effects are not uncommon since soil available N can either decrease when drought-induced reduction in N mineralization rate is higher than drought-induced reduction in plant N uptake (He and Dijkstra, 2014; Jiao et al., 2016; Sanaullah et al., 2012; Sardans and Penuelas, 2012) or increase when, inversely, reduction in plant N uptake is higher than the decrease of N mineralization rate (Dijkstra et al., 2015; Mariotte et al., 2017). The structural equation modelling for N parameters confirms such

results by highlighting the strong N demand from plants at Site 2, while at Site 1 soil available N tended to be used by microbes rather than by plants.

However, shifts in soil available N in response to soil moisture reduction had marginal (Site 1) or less (Site 2) impact on forage NNI compared to direct negative effects of soil moisture reduction. Therefore, the decrease in forage NNI under drought at both grassland sites seems primarily related to water shortage that induced lower transpiration and water fluxes, and thus lower mass flow of N to roots, stems and shoots. Such direct effects of soil moisture reduction are complemented by indirect negative effects, through reduction in soil N availability, only at Site 2, which is the site where plants tend to be more N-limited (forage N:P ratio of 6.8) and where forage NNI values were the most reduced in response to drought (Fig. 3c,f). This contradicts our third hypothesis for what concerns forage N status since the indirect path from soil moisture reduction to forage N status through soil N was -0.20 at the less fertile soil, while it was -0.56 at the more fertile soil. As such, our findings are consistent with those of a previous study (Hofer et al., 2017) and suggest that drought-induced effect on forage NNI partly depends on the degree of N limitation for plants (i.e., site-specific).

In contrast to soil N, reduction in soil moisture consistently induced a decrease in soil P at both sites (Fig. 4a,d), but the amplitude of these effects was relatively low, despite the large difference in soil available P between both sites (9 mg kg^{-1} at Site 1 compared to 51 mg kg^{-1} at Site 2). At both sites, the structural equation modelling of the P parameters confirms the strong negative impact of soil moisture reduction on soil P availability. However, reduction in soil P was not the main mechanism involved in drought-induced reduction in forage P. The forage P decreased with decreasing soil moisture, with stronger effects at Site 2 (i.e., P-rich soil) compared to Site 1 (i.e., P-limited soil). Interestingly, the mechanisms involved in drought-induced reduction in forage P differed between both sites. At Site 2, forage P was directly impacted by soil moisture reduction, indicating a decrease in P uptake due to water

restriction. Indeed, diffusion is the major process moving P to the roots for uptake (Schachtman et al., 1998) and strongly decreases under drought (Bhadoria et al., 1991), thus reducing even more P mobility and plant P uptake. Moreover, soil available P is mostly located in the upper cm of soil (Lambers et al., 2008), which further reinforces the effect of drought. By contrast, at Site 1, forage P was not significantly impacted by direct effect of soil moisture reduction but rather by indirect effects through decrease in microbial P (indirect path = -0.43). Indeed, microbes were important actors of the P cycle at Site 1, and we found a positive effect of microbial P on forage P (path = 0.15). This suggests transfer of P from microbes to plants. P-limited soils with low pH, such as in Site 1, generally promote symbiosis between plants and mycorrhizal fungi, because the latter can considerably improve plant P nutrition. Indeed, arbuscular mycorrhizal fungi can both release oxalic acid to induce the release of phosphorus adsorbed on soil (Jansa et al., 2011) and extend threadlike hyphae into the soil to enhance the uptake of phosphorus (Hodge, 2017). We suggest that the microbial pathway involved in the P cycling is partly, if not fully, related to arbuscular mycorrhizal fungi. Additional analysis of soil microbial communities through high-throughput sequencing (see Schenkel et al., 2019) highlights higher abundance of arbuscular mycorrhizal fungi (Glomeromycetes family) at Site 1 (261.6 OTUs) than at Site 2 (183.1 OTUs) and supports our hypothesis. Overall, our findings reveal the strong influence of soil P availability in the processes involved in drought-induced reduction of forage P and validate our third hypothesis concerning the stronger effect of drought on competition between plants and microbes at the less fertile site.

5. Conclusions

Forage quantity and quality responses to drought reflect various processes affecting nutrient cycling, and highlight the complex relationships among soil, microbes, and plants, with more intense impacts on P than on N parameters. Our results suggest that the two grasslands suffered mainly from water shortage *per se*, and to a lesser extent from drought-induced nutrient deficiency (P more than N), thus pointing out that higher fertilization cannot alleviate the impacts of drought. In order to secure a sustainable and resilient forage production, future management schemes should not only rely on agronomic aims (production of quality forage), but also consider the occurrence of these extreme drought events. Under the climatic conditions of central Europe, our results show that a late-season drought represents probably a greater threat to forage production than an early-season drought. For this reason, when suitable, irrigation should receive priority during these periods (Hopkins and Prado, 2007). The timing of mowing has also gained new importance and, in periods of increasing water shortage, measures should be primarily focused on adaptive management, such as reducing mowing frequency and/or adapting cutting height (e.g., Deléglise et al., 2015). Importantly, the characteristics of the site can play an important role in predicting the response of grasslands to drought and consequent changes in forage quantity and quality. Management practices should therefore be better adapted to cope with interactive effects of drought and site-specific conditions of soil fertility, microbial community composition (i.e., mycorrhizal abundance) and nutrient limitation for plants.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.agee.2019.04.027>.

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